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Isolation and screening of alkaline protease producing bacteria and induction of overproducing *Bacillus licheniformis* mutants through UV irradiation

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ABSTRACT

Screening was done from soil samples collected from black cotton soil, groundnut field, milk processing unit, Kotappakonda hill area in Guntur Dist,and Tirumala in Chittur District, A.P. Indian soils are best for alkaline protease producing bacteria that resulted in isolation of 21 alkaline protease producing alkaliphilic bacterial strains. MS6 showed the highest production (110U/ml) after 48h. This isolate was identified as *Bacillus licheniformis* by Microbial Type Culture Collection center (MTCC) and gene bank, Institute of Microbial Technology (IMTECH), Chandigarh, India. This strain was used to examine the changes in alkaline protease production following UV irradiation. Induction of mutation in *Bacillus licheniformis* strain was carried out by 0, 3, 6, 9, 12, 15, 18 and 20 min with 30-W germicidal lamp that has radiation at 2540 - 2550A^0 at a distance of 15cm in dark and irradiated. A total of 17 mutants were selected. They were designated as Bl1 to Bl9 and Bl10 to Bl17. Among these only three strains viz., Bl2, Bl11, and Bl16 did exhibit high efficiency in production on the basis of relative growth production (C/G). Of the seventeen mutants of *Bacillus licheniformis*, ten were chosen to assay their productivity. Mutants no Bl8, Bl3, Bl16 were the most effective in enzyme production under submerged conditions being 180, 140,128U/ml respectively. Results revealed that alkaline protease activity assay under submerged culture conditions was more accurate than the relative growth production (C/G) method because there is no correlation between zone diameter and the ability to produce the enzyme in submerged cultures. High level of productivity increased with Bl8 mutant of *B.licheniformis*, indicating that the enzyme is to be thermo-alkaliphilic protease.

Keywords: Alkaline protease, isolation and screening, UV irradiation, mutants.

INTRODUCTION

Proteases are enzymes which catalyze the hydrolysis of peptide bonds. Microorganisms are capable of producing these enzymes intracellularly and extracellularly. The isolation of proteases especially the extracelluar proteases of microbial origin is easy and economical. Seasonal fluctuations in the availability of raw material usually do not affect the enzyme production by microbes. There are possibilities for genetic and environmental manipulations to improve yield and properties of the enzymes. Alkaline proteases are referring to proteolytic enzymes which work optimally in alkaline pH (Barett, 1994; Gupta *et al*., 2002). The vast diversity of proteases, in contrast to the specificity of their action has attracted worldwide attention in attempts to exploit their physiological and biotechnological applications (Fox *et al*.,
1991; Poldermans, 1990) like food and feed industry, peptide synthesis, leather industry, management of industrial household waste, photographic industry, medical usage, silk gumming and detergents industry etc.
The naturally occurring alkaline environments comprise alkaline soils, soda lakes, alkaline springs etc. Isolation and screening of bacteria from these natural environments can be supposed to be useful for obtaining bacterial strains with the potential of yielding alkaline protease. Strain improvement is an essential part of process development for fermentation products. Improved strains can be achieved by inducing genetic variation in the natural strain and subsequent screening. In recent years new procedures such as rational screening and genetic engineering have begun to make a significant contribution to this study but mutagenesis and selection- so-called random screening is still cost effective procedure, and reliable short term strain

development is frequently the method of choice (Rowlands, 2002; Iftikhar, 2010). Mutagenic procedures can be optimized in terms of type of mutagen and dose. Ultraviolet radiation is one of the well known and most commonly used mutagen. It is universally used to induce genetically improved strains.
The aim of this study was isolation and screening of high yielding alkaline protease producing bacteria from different alkaline environments and to examine the effect of U.V irradiation on alkaline protease production and induction of over producing strains. The promising strains in this study may be used in various economic industrial applications.

MATERIALS AND METHODS

Isolation of bacterial strain

Alkaliphilic proteolytic bacteria were isolated form alkaline soil samples collected from black cotton soil, groundnut field, milk processing unit, Kotappakonda hill area in Guntur Dist,and Tirumala in Chittur District, A.P. These soil samples were suspended in water by vigorous vortexing and serial dilutions were made upto 10^{-9} in sterile water. 0.1 ml of appropriate dilution was added to petri plate on skim milk agar containing pH 9.5 and incubated at 40^0C for 24h. A clear zone of skim milk hydrolysis around the colonies indicated alkaline protease production by the organism. These colonies were picked and purified by streaking on skim milk agar. The purified proteolytic isolates were stored and maintained in nutrient agar slants (pH 9.5) by sub culturing at monthly intervals. More than 20 isolates were thus collected.

Screening of the isolates for the alkaline Protease production

The proteolytic bacterial strains were screened for the yield of alkaline protease by submerged fermentation in Erlenmeyer flasks with the production medium (GYP) (Kumar and Bhalla, 2004) containing (G/L) Glucose 10, Yeast extract 5, Peptone 5, $MgSO_4$ 7.H_2O 0.2, K_2H PO_4 1, pH of the medium was adjusted to 9.5 with 1N NaOH or 1N HCL and inoculated with 10% (v/v) of 24h old seed culture prepared in the nutrient broth at 40^0C for 48h on a rotary shaker at 70rpm. After incubation the culture broth was centrifuged at 10,000 rpm for 20min. The supernatant was used as the crude enzyme for the assay of alkaline protease activity.

Enzyme assay

According to Udandi Boominadhan *et al.*, (2009), the enzyme was assayed in the reaction mixture containing 2.0 ml of 0.5% casein solution in 0.1M Carbonate – Bicarbonate buffer pH 9.5 and 1ml enzyme solution in a total volume of 3.0ml. Reaction mixture was incubated for 5 min at 40^0C. The reaction was terminated by adding 3ml of 10% ice-cold trichloroacetic acid. The tubes were incubated for one hour at room temperature. Precipitate was filtered through whatman no.1 filter paper and the filtrate was collected. For the color development for the assay of tyrosine in the filtrate, 5ml of 0.4 M Sodium carbonate and 0.5 ml of Folin phenol reagent were added to 1ml of filtrate, vortexed immediately, incubated for 20 min at room temperature and OD was taken at 660 nm. Concentration of tyrosine in the filtrate was read from a standard curve for tyrosine already prepared. One unit enzyme activity was taken as the amount of enzyme producing 1µg of tyrosine under standard assay conditions and expressed as units ml^{-1} enzyme.

Identification of the high yielding strain

Various Cultural, Morphological and Biochemical properties of the high yielding strain were studied.

Bacterial strain and culture condition

Newly isolated *Bacillus licheniformis* was employed in the present study. Culture of *Bacillus licheniformis* was grown at 40^0C for 24 hrs in nutrient broth at 70 rpm on an incubatory shaker, at pH 9.5. The contents of the flasks were centrifuged at 4000 rpm for 10min and the supernatant was decanted. The cell pellets were washed thoroughly with sterile saline (0.9% NaCl) followed by sterile distilled water. Finally the cell mass was suspended in sterile saline

and used as a source of cell suspension for irradiation.

U.V mutagenesis

UV irradiation as physical mutagenic agent was used for over production of alkaline protease. Mutagenesis was carried out according to Justin *et al*., (2001) using different exposure times and radiation intensities i.e., distances from irradiation source. Two ml of this suspension was placed into 15 cm diameter glass petridishes at a distance of 15 cm in dark and irradiated for 0, 3, 6, 9, 12, 15, 18 and 20 min. A 30-W germicidal lamp that has about 90% of its radiation at 2540 - 2550A^0 UV was used. The treated samples were transferred into sterile test tubes covered with a black paper and kept in the refrigerator over night to avoid photo reactivation. 0.5 ml of suitable dilution of *Bacillus licheniformis* was spread on eight skim milk agar media plates at pH 9.5 and incubated for 24h at 40^0C. Colonies developed after incubation were counted and transplanted onto slants. The numbers of survivals from each exposure are estimated. The U.V survival curves are plotted. Plates having between 10 and 0.1% survival rate were selected for the isolation of mutants. The isolates were selected on the basis of macroscopic differential characteristics. Mutation frequency was mentioned to be high when the survival rates were between 10 and 0.1% (Hopwood *et al.*, 1985).

Enzyme detection

Skim milk agar was used to detect the alkaline protease production according to Kunammeni *et al.*, (2003). Sterile skim milk was added after autoclaving and cooling the medium at 40^0C. In situ protease production was demonstrated by the clearing of opaque milk proteins in the area surrounding colonies growing on the surface.

Isolation of alkaline protease over producing mutants and enzyme assays

According to Solaiman *et al.*, (2004), for isolation of overproducing mutants after U.V irradiation developed colonies were inoculated into skim milk agar medium under alkaline conditions and incubated at 40^0C for 24h. Depending upon the zone of clearance mutants with high activity were selected on the basis of the relative growth production (C/G) of the bacterial colonies. Three measurements were used to determine the enzyme assay. They are,

1. Enzyme production defined as lyses zone area mm^2 (C)
2. Colony Growth area mm^2 (G) and
3. Relative growth production

The enzyme activity of superior alkaline protease producing mutants was assayed according to the method of Udandi Boominadhan *et al.*, (2009) using GYP medium (Glucose yeast extract peptone medium). One unit of the enzyme activity is defined as amount of enzyme producing 1µg of tyrosine under standard assay conditions and expressed as units ml^{-1} of enzyme.

RESULTS AND DISCUSSION

Isolation and screening of bacterial cultures for alkaline protease production

21 bacterial cultures, capable of producing alkaline protease were selected and screened and the results are presented in Table I. The 21 bacterial cultures produced alkaline proteases at varying levels from 44Uml^{-1} to 110 Uml^{-1}. Among the cultures tested the culture number MS6 obtained from alkaline soil of the milk processing unit gave the maximum yield of 110 Uml^{-1}, was selected and identified as *Bacillus licheniformis* by Microbial Type Culture Collection center (MTCC) and gene bank, Institute of Microbial Technology (IMTECH), Chandigarh, India. The results are shown in Table II. Casein agar used in this study was having pH 9.5, the isolates obtained could be alkalophiles, either facultative or obligate. The use of alkaline casein or milk agar for the isolation of alkaline protease producing bacteria has been reported by some workers (Durham *et al.*, 1987; Nihalani and Satyanarayana, 1992; Gessesse and Geshe, 1997). Since the incubation was performed at 40^0C, the isolates obtained may either be thermo tolerant or thermophilic. So the method used in this study can be considered as an

easy and simple for the isolation of proteolytic strains which could possibly be the good source of thermo stable alkaline proteases. Before selecting the MS6 strain for further studies its ability to maintain the high yielding nature was studied by sub culturing and testing the yields at monthly intervals. Repeated sub culturing has not affected the yield of the strain.

Detection, isolation of UV mutants and their alkaline protease activity

The wild strain MS6 (*Bacillus licheniformis*) was subjected to strain improvement. The numbers of survivals from each exposure are represented in Table III. A total of 17 mutants were selected and were designated as Bl1 to Bl9 and Bl10 to Bl17 respectively. Among these only three strains viz., Bl2, Bl11, and Bl16 did exhibit high efficiency in production on the basis of relative growth production (C/G). Table IV indicates that the C/G values ranged from 3.72 for mutant no Bl5, 49.24 for Bl2 which was 7.84 times more active than the original strain (wild type). Of the seventeen mutants of *Bacillus licheniformis,* ten were chosen to assay their productivity according to Udandi Boominadhan *et al.,* (2009). The results are tabulated in Table V and fig 1. Results proved that there is no correlation between zone diameter and the ability to produce the enzyme in submerged culture. Bl2 produced 49.24 in relative growth production while mutant produced 120Uml^{-1} of alkaline protease. On the other hand, Bl3 with 19.6 G was relatively more efficient in enzymatic activity (140 Uml^{-1}) than Bl2. The area of the hydrolysis on casein agar (Aunstrup, 1974) and the ratio of colony size to casinolysis (Niheter *et al*., 1986) have not been found reliable to assess *Bacillus licheniformis.*

Mutants showed different responses to UV radiation for alkaline protease production and these variations are more probably due to the differences induced in their genetic background. Therefore, results obtained revealed variation in gene expression i.e. enzyme activity as reported by Justin *et al.,* (2001). It is suggested that the increase in enzyme productivity might result from damage of genes located on plasmids which have a negative influence on the chromosomal alkaline protease production genes i.e. repression as reported by Solaiman *et al.,* (2003). Variations may be also due to some factors like damage or mutation occurring in genes and differences in their ability of repairing their DNA. The repair enzymes themselves might be damaged and the repair mechanism is not universal. So replication cannot take place again (Ben, 2003). In the present study, among the seventeen UV mutants isolated, the relatively more efficient strain for protease production was Bl8. Repeated sub culturing was not affecting the yield by mutant Bl8 of *B.licheniformis.*

Conclusion

From the present results, it is concluded that the protease produced by the test organism i.e., *Bacillus licheniformis* owing to its alkaline nature and stability. Mutants of this strain especially Bl8 is recommended for various applications.

Table I: Screening of the isolates for the alkaline protease production

S.No	Isolate number	Enzyme activity Uml^{-1}
1	BS1	82
2	BS2	85
3	BS3	82
4	BS4	90
5	GS1	94
6	GS2	78
7	GS3	92
8	KS1	100
9	KS2	100
10	KS3	104
11	KS4	54
12	MS1	44
13	MS2	92
14	MS3	100
15	MS4	104
16	MS5	100
17	MS6	110
18	TS1	42
19	TS2	54
20	TS3	72
21	TS4	78

Table II: Morphological, Physiological and Biochemical Tests for Strain Number MS: 6 Morphological Tests

Tests	Results
Colony Morphology	**MS-6**
Configuration	Round
Margin	Wavy
Elevations	Convex
Surface	Smooth
Density	Opaque
Pigments	Cream
Gram's Reaction	+
Shape	Rods
Size	Moderate
Arrangement	Chains
Spore	
Endospore/granules	+
Position	Sub terminal
Shape	Oval
Sporangia Bulging	-
Motility	-
Florescence(UV)	-

Table II: (Contd.) Physiological Tests

Tests	Results
Growth at Temp.	**MS-6**
4^0C	-
10^0C	+
15^0C	+
25^0C	+
30^0C	+
37^0C	+
42^0C	+
55^0C	+
65^0C	-
Growth at pH	-
5.0	+
5.7	+
6.8	+
8.0	+
9.0	+
11.0	+
Growth on NaCl (%)	
2.5	+
5.0	+
7.0	+
8.5	+
10.0	+
Growth Under Anaerobic	+/-

Table II: (Contd.) Biochemical Tests

Tests	Results
	MS-6
Growth on MacConkey Agar	-
Indole Test	-
Methyl Red Test	-
Voges Proskauer Test	-
Citrate Utilization	+
Gas Production from Glucose	-
Casein hydrolysis	+
Starch Hydrolysis	+
Urea Hydrolysis	-
Nitrate Reduction	+
H_2S Production	-
Cytochrome Oxidase	+
Catalase Test	+
Oxidation/ Fermentation (O/F)	F
Gelatin Hydrolysis	+
Arginine dihydrolase	+
Lysine decarboxylase	-
Ornithine decarboxylase	-

Table II: (Contd.) Acid Production from carbohydrates

Tests	Results
	MS-6
Adonitol	-
Arabinose	+
Cellobiose	-
Dextrose	+
Dulcitol	+
Fructose	+
Galactose	-
Inositol	-
Lactose	-
Maltose	+
Mannitol	-
Melibiose	-
Raffinose	-
Rhamnose	+
Salicin	-
Sorbitol	-
Sucrose	+
Trehalose	+
Xylose	-

On the basis of above tests, the organism has been identified as follows:

S.No.	Strain Designation	Identity	MTCC No.
1.	MS-6	*Bacillus licheniformis*	10,008

Table III: Survival of *Bacillus licheniformis* after UV exposure using different doses at distance of 15cm

Irradiation time (min)	**Number of cells/ml after irradiation**	**Survival Percent**	**Kill percent**
0(control)	21.2×10^7	100	0
3	150×10^6	70.75	29.25
6	80×10^6	37.74	62.26
9	500×10^5	23.58	76.42
12	180×10^5	8.49	91.51
15	800×10^4	3.77	96.23
18	600×10^4	2.83	97.17
21	2000×10^3	0.94	99.06

Table IV: The alkaline protease production (C/G) of the *Bacillus licheniformis* Mutants

Mutant No.	G	C	C/G
Bl1	31.4	706.50	22.50
Bl2	12.5	615.44	49.24
Bl3	19.6	379.94	19.38
Bl4	38.47	907.46	23.59
Bl5	13.5	50.24	3.72
Bl6	0.79	0.00	0.00
Bl7	3.14	12.56	4
Bl8	20.0	803.84	40.19
Bl9	12.5	78.50	6.28
Bl10	19.6	254.36	12.98
Bl11	12.5	452.16	36.17
Bl12	7.07	200.9	28.42
Bl13	19.6	157.0	8.01
Bl14	19.6	146.0	7.45
Bl15	20.2	410.7	20.54
Bl16	7.07	260.9	36.90
Bl17	12.5	379.94	30.40
Wild type	12.5	78.50	6.28

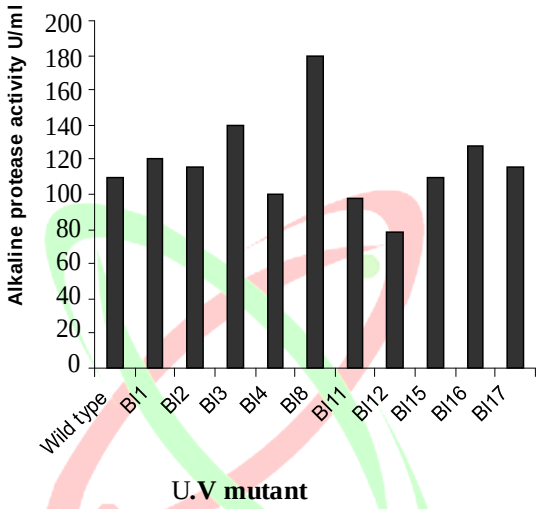
G: Colony growth area (mm^2)

C: Enzyme production defined as lyses zone area (mm^2)

Table V: The alkaline protease activity of the highest *Bacillus licheniformis* mutants

Mutant No.	C/G	Enzyme Activity Uml^{-1}
Wild type	6.28	110
Bl1	22.50	120
Bl2	49.24	116
Bl3	19.38	140
Bl4	23.59	100
Bl8	40.19	180
Bl11	36.17	98
Bl12	28.42	78
Bl15	20.54	110
Bl16	36.90	128
Bl17	30.40	116

Fig 1U.V mutants from *Bacillus licheniformis* and their alkaline protease activity



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